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Growth hormone induces the tyrosine phosphorylation and nuclear accumulation of components of the ISGF3 transcription factor complex

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Abstract

Growth hormone induced the accumulation of the stat91 and p84 subunits of the transcription factor complex ISGF3 in nuclear fractions of 3T3-F442A cells. Nuclear levels of p84 and stat91 peaked 30–60 min after addition of growth hormone. Growth hormone also induced the tyrosine phosphorylation of two proteins, with similar sizes to stat91 and p84, in both nuclear and cytosolic fractions. The time course of growth hormone-induced tyrosine phosphorylation of these proteins paralleled the nuclear accumulation of stat91 and p84. Immunoprecipitation with stat91-specific antibodies confirmed that growth hormone induced the tyrosine phosphorylation of stat91 and an associated protein of $M_r \approx 120$ kDa. These findings suggest a mechanism for the modulation of specific gene transcription by growth hormone.

Key words: Growth hormone; 3T3-F442A cell; ISGF3; Tyrosine phosphorylation; Transcription factor

1. Introduction

The effects of pituitary growth hormone (GH) on tissue growth, metabolism and development are well documented [1,2]. Only recently, however, significant details have emerged concerning the molecular basis of these actions. The GH receptor is a member of the cytokine superfamily of receptors [3] which, though devoid of intrinsic tyrosine kinase activity, promote rapid changes in intracellular protein tyrosine phosphorylation [4–7]. Carter-Su and colleagues were first to demonstrate tyrosine phosphorylation of the GH receptor in response to GH [8] and, more recently, showed that the receptor associates with JAK2 [9], a member of the Janus family of tyrosine kinases [10]. Apart from the GH receptor, the substrates for JAK2 are not known and it is not clear if JAK2 is involved in transmitting all or only a subset of GH signals.

In common with most hormones, growth factors and cytokines, GH activates the signalling cascade involving mitogen-activated protein (MAP) kinase and $p90^{rsk}$ [11–14]. Since both of these enzymes translocate to the nucleus and phosphorylate various transcription factors [15], this cascade is potential mechanism for the modulation of gene transcription by GH. Recently, a second, more direct, mechanism for signalling to the nucleus has been elucidated. Originally described for interferon α/β [16–18], this involves the multimeric transcription factor ISGF3. Upon cell stimulation components of the complex become tyrosine phosphorylated in the cytosol before translocating to the nucleus where they interact with the response elements for specific target genes. It appears that one component of ISGF3, a 91-kDa protein known as stat91, is a common target for many other cytokines and growth factors, including interferon-γ [19–22], interleukin-6 [21,23], interleukin-10 [19], epidermal growth factor [20,21,24,25] and platelet-derived growth factor [25]. Stat91 can combine with other proteins in the nucleus and this governs its DNA binding specificity [26]. Previously we reported [13] that multiple proteins undergo rapid tyrosine phosphorylation in response to GH in 3T3-F442A preadipocytes. In the present study we have investigated whether stat91 undergoes increased tyrosine phosphorylation and nuclear translocation in response to GH.

2. Materials and methods

2.1. Materials

Protein A-Sepharose was obtained from Pierce, Chester, UK. Polyclonal antibodies to phosphotyrosine were obtained from ICN. A polyclonal antibody to stat91 was a generous gift from Dr Andrew Larner (NIH, MD, USA). A monoclonal antibody, anti-p84/stat91, which recognises both the 84-kDa and 91-kDa (stat91) components of ISGF3 was obtained from Affiniti Research, Nottingham, UK. All other chemicals were obtained from Sigma.

2.2. Cell culture, treatment and preparation of subcellular fractions

3T3-F442A cells (provided by Dr. Howard Green, Harvard Medical School) were cultured and treated with 25 nM human pituitary GH (obtained from NIADDK, Bethesda, USA) as described previously [13]. Nuclear fractions were prepared using a modification of the method described by Sadowski and Gilman [27]. Cells from twenty 100-mm plates were suspended in 2.5 ml of Buffer A (20 mM HEPES pH

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7.9, 20 mM Na fluoride, 1 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM Na orthovanadate, 1 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl fluoride, leupeptin (2 μg/ml), aprotinin (2 μg/ml) and pepstatin A (2 μg/ml)) containing 0.1% NP40. The cells were allowed to swell on ice for 10 min and were then broken by passage through a 26G needle. The lysate was centrifuged at 1500 rpm for 4 min in a microfuge yielding a nuclei-enrichted pellet and a post-nuclear supernatant. The pellet was resuspended in Buffer A (without NP40) and re-centrifuged. The resulting nuclear pellet was resuspended in 250 μl of Buffer B (Buffer A containing 20% glycerol and 420 mM NaCl) and incubated on ice with occasional agitation for 60 min. The suspension was then centrifuged at 14,000 rpm for 20 min in a microfuge and the resulting supernatant taken as the nuclear extract. Cytosolic extracts were prepared by centrifugation of the post-nuclear supernatant at $1000 \times g_{max}$ for 10 min followed by centrifugation of the resulting supernatant at $200{,}000 \times g_{max}$ for 30 min.

2.3. Immunoprecipitation

Cells from ten 100 mm plates were scraped into 1 ml of i.p. lysis buffer (25 mM HEPES pH 7.4, 50 mM NaCl, 5 mM EDTA, 30 mM Na pyrophosphate, 50 mM Na fluoride, 1 mM Na orthovanadate, 10% glycerol, 1% Triton X-100 and protease inhibitors as above) and rotated at 4°C for 20 min. The lysates were clarified by centrifugation at 14,000 rpm in a microfuge. For immunoprecipitation of stat91, equal quantities of protein from control or GH-treated cells were incubated with anti-stat91 (1:100 dilution) for 3 h and precipitates collected by incubation for 1 h with protein A-Sepharose. Immunoprecipitates were washed three times with i.p. lysis buffer and once with Tris-buffered saline and finally boiled with SDS sample buffer. Samples were stored at −20°C until use.

2.4. Western blotting

Samples were electrophoresed on 8% SDS-PAGE followed by transfer to nitrocellulose at 125 mA for 16 h. Blots were blocked in a solution containing 3% bovine serum albumin and probed with anti-stat91, anti-p84/stat91 or anti-phosphotyrosine antibodies. Phosphotyrosine blots were developed with [^{125}I]protein A (ICN) and stat91 blots with the enhanced chemiluminescence system (ECL, Amersham). All data presented are representative of experiments done on at least three separate occasions.

3. Results and discussion

In resting cells stat91 resides predominently in the cytosol. To act as a transcription factor cytosolic stat91 is activated by tyrosine phosphorylation before translocating to the nucleus [26]. To investigate whether stat91 is involved in GH signalling, we first examined whether GH induced its accumulation in nuclei-enriched fractions. We used an antibody which specifically recognises both stat91 and p84, which are alternatively spliced products of the same gene [28]. Immunoblots revealed that stimulation of 3T3-F442A cells with GH increased the levels of both stat91 and p84 in nuclear fractions (Fig. 1). Maximal accumulation occurred between 30 and 60 min following addition of GH. In contrast, the cytosolic levels of stat91 and p84 were not consistently changed by GH treatment (Fig. 1).

We next examined whether GH could induce the appearance of tyrosine phosphorylation proteins in the nucleus. Phosphotyrosine immunoblots revealed that GH promoted tyrosine phosphorylation in nuclei-enriched fractions, most notably of a protein with a similar size to stat91 (Fig. 2a). A faintly discernible protein ($M_r \approx 84$ kDa) exhibited a similar time course of GH-induced tyrosine phosphorylation (Fig. 2a). 91-kDa and 84-kDa phosphotyrosyl proteins were also detected in cytosolic fractions from GH-treated cells (Fig. 2b). The fact that the time course of GH-induced tyrosine phosphorylation of the 91- and 84-kDa proteins detected in the nucleus closely paralleled the accumulation of stat91 and p84 in the same fraction strongly suggests that these phosphotyrosyl proteins are the ISGF3 subunits stat91 and p84.

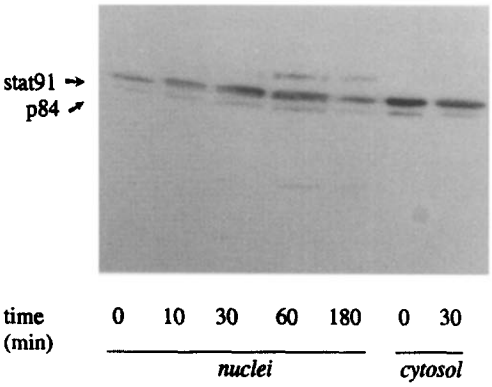

Fig. 1. Time course of growth hormone-induced nuclear accumulation of p84 and stat91. Cells were left untreated (time 0) or stimulated with 25 nM GH for the indicated times. Nuclear extracts were prepared according to section 2 and equal quantities (100 μg) of protein immunoblotted with anti-p84/stat91 antibodies. Cytosolic extracts (50 μg protein) from untreated or GH-treated (30 min) cells were run in parallel for comparison. The migratory positions of p84 and stat91 are indicated.

Further evidence that GH induces the tyrosine phosphorylation of stat91 was obtained by immunoprecipitation with a polyclonal antibody specific for stat91 followed by phosphotyrosine immunoblotting. Fig. 3 shows that a phosphotyrosyl-protein with an apparent molecu-

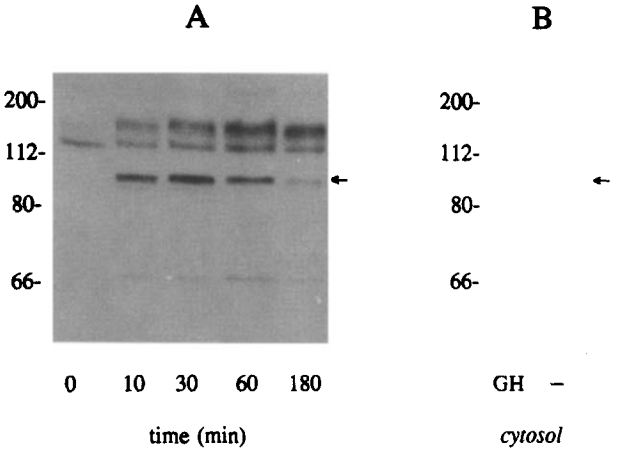

Fig. 2. Time course of growth hormone-induced protein tyrosine phosphorylation in nuclear fractions. Cells were left untreated (time 0) or treated with 25 nM GH for the indicated times. Nuclear extracts (panel A) or cytosolic extracts (panel B) were prepared according to section 2 and 100 μg of protein immunoblotted with anti-phosphotyrosine antibodies. The migratory positions of molecular mass markers are shown to the left. The 91-kDa protein exhibiting increased tyrosine phosphorylation in response to GH is arrowed.

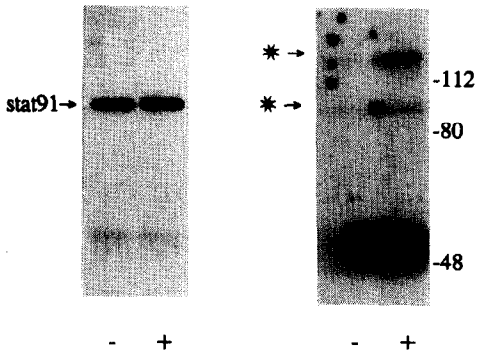

Fig. 3. Growth hormone induces the tyrosine phosphorylation of stat91. Right panel: lysates from control (−) or GH-treated (+) cells were immunoprecipitated with anti-stat91 antibody and probed for phosphotyrosine as described in section 2. The positions of two proteins exhibiting an increase in tyrosine phosphorylation in response to GH are indicated by an asterisks. Left panel: Parallel anti-stat91 immunoprecipitates immunoblotted with stat91 antibodies.

lar mass of 91 kDa was detected in anti-stat91 immunoprecipitates from GH-treated but not control cells. Immunoblotting of parallel immunoprecipitates with stat91 antibodies confirmed the migratory position of stat91 and that similar quantities of stat91 were present in each sample (Fig. 3). Qualitatively similar results were obtained using the stat91/p84 monoclonal antibody for immunoprecipitation (data not shown). However we were unable to detect GH-induced tyrosine phosphorylation of p84 in these experiments. This may be a sensitivity problem since this antibody detects much lower levels of p84 compared to stat91 on immunoblots of 3T3-F442A cell lysates (data not shown). Furthermore the data in Fig. 2 indicates that the GH-induced tyrosyl phosphorylation of p84 is less than that of stat91.

Interestingly, a second phosphotyrosyl-protein ($M_r \approx$ 120–130 kDa) was consistently detected in both anti-stat91 (Fig. 3) and stat91/p84 immunoprecipitates (data not shown), suggesting that, at least in GH-treated cells, this protein is tightly associated with stat91. Because of its size, it is tempting to speculate that this protein is either the GH receptor or JAK2, both of which undergo GH-stimulated tyrosine phosphorylation [9]. This possibility is at present under investigation.

In conclusion we have shown that GH induced the tyrosine phosphorylation and nuclear accumulation of the transcription factor stat91. In addition, we observed GH-induced nuclear accumulation of p84 and tyrosine phosphorylation of a similar sized protein, suggesting that GH may target other components of ISGF3. Variations in the tyrosine phosphorylation and assembly of ISGF3 subunits in response to different cell stimuli have been proposed to confer specificity by directing interaction with different DNA response elements [26]. Although the precise details of this will require further study, our data describe a further mechanism for the regulation of specific gene transcription by GH.

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